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# Saprophytic yeasts: effective biocontrol agents against *Aspergillus flavus*

Afsah-Hejri, L.

*Food Safety Research Center (FOSREC), Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

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**Abstract**

Aflatoxins are carcinogenic, mutagenic and teratogenic fungal toxins predominantly produced by *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*). Members of the *Aspergillus* family are wound-invading pathogens that can infect pistachio trees and nuts. The pistachio nut is a favorite tree nut worldwide, and more than half of the world's pistachio production is from Iran. Pistachio nuts can easily be infected with *Aspergillus* spp. due to early splitting or due to animal, insect or physical damage. Any established infection of *Aspergillus* under high relative humidity and temperature results in the production and rapid accumulation of aflatoxins in pistachio nuts. It is impractical to remove aflatoxins from pistachio nuts after they are produced. Some microorganisms (such as saprophytic yeasts) have been reported to have an antagonistic effect against *Aspergillus* spp. This study aimed to isolate saprophytic yeasts from pistachio fruits and leaves and investigate their biocontrol activities against a toxigenic strain of *Aspergillus flavus* (*A. flavus*). Saprophytic yeasts were identified based on their morphological properties and biochemical tests. In total, 24 yeast isolates were obtained from pistachio fruits and leaves, and their antagonistic effect on *A. flavus* (PTCC 5006) was investigated. Five saprophytic yeast isolates, displaying the highest biocontrol activities against *A. flavus* (PTCC 5006), were identified as *Pseudozyma fusiformata*, *Cryptococcus albidus*, *Rhodotorula fragaria*, *Cryptococcus hungaricus* and *Rhodotorula hinula*. The biocontrol activities of these yeast isolates were evaluated by their inhibitory effects on sporulation, colony expansion, biomass production and prevention of aflatoxin B1 (AFB1) production. *Pseudozyma fusiformata* was the most effective yeast isolate in terms of spore reduction (84.6%) and inhibition of AFB1 production (89.1%). *Cryptococcus albidus* produced the maximum reduction in fungal dry weight (77.9%). Based on these results, isolated saprophytic yeasts from pistachio fruits and leaves can be used as effective biocontrol agents against the growth of *Aspergillus* and aflatoxin production.

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## Introduction

The pistachio nut (*Pistachia vera* L.) is a favorite tree nut worldwide. Pistachio trees are widely cultivated in saline, hot and dry areas (Metheney *et al*., 1998) of Mediterranean countries, the Middle East and the USA. Iran has a significant share of the worldwide pistachio production, followed by the USA, Turkey, Syria, China and Greece (FAOSTAT, 2008; Zheng *et al*., 2012) and the majority of Iran's pistachio production is from Kerman, Rafsanjan. Pistachio nuts substantially contribute to the agricultural exports of Iran and are known as "green gold" in Iran (Aghdaie, 2009). Pistachio kernels are a rich source of oil and essential fatty acids for humans (Maskan and Karatas, 1999). Depending on the variety, the oleic acid content of pistachio kernels varies between 51.60-67.86%, with linoleic acid contents between 11.56-27.03% in pistachio kernels (Tsantili *et al.*, 2010). Pistachio kernels contain an average protein content of 20% (Tsantili *et al*., 2010) making them a good source of protein that can be added to snacks and confectionary products.

Mycotoxins are secondary metabolites produced by filamentous fungi that are distributed worldwide and can contaminate a variety of foodstuffs. Mycotoxin contamination of food and feed is a serious health risk for humans and animals and has been raised as a high priority food safety issue during the past three decades (Afsah-Hejri *et al*., 2013a). Mycotoxin contamination of food and feedstuffs results in a significant economic loss both for producers and handlers (Herrman *et al.*, 2002). Such negative impacts predominantly affect developing countries where pre-harvest, harvest and post-harvest techniques are not efficient enough to prevent the growth of contaminating fungi (Rustom, 1997). Among all mycotoxins, aflatoxins (AFs) are the most important groups and attract a significant amount of attention due to their mutagenic, carcinogenic and teratogenic effects (Bhat *et al.*, 2010). In warm and hot climates, AFs are predominantly produced by

*Corresponding author.
Email: *l.afsahhejri@gmail.com*

*Aspergillus* spp., such as *Aspergillus parasiticus* (*A. parasiticus*) and *Aspergillus flavus* (*A. flavus*) (Afsah-Hejri *et al*., 2013a, 2013b). Aflatoxin $B_1$ ($AFB_1$) is the most toxic aflatoxin with the highest carcinogenic effects (Afsah-Hejri *et al*., 2011), and the presence of AFs in food and feedstuffs imposes serious health risks to humans and animals (Leszczynska *et al*., 2000).

*Aspergillus* family members are wound-invading pathogens. Pistachios can be easily damaged by early splitting, birds, insects or mechanical damage during harvest (Mahoney and Rodriguez, 1996). As a result of early splitting, damaging or cracking of the pistachio nut shell, *Aspergillus* spores can enter and infect the kernel (Sommer *et al*., 1986). *Aspergillus* spores enter through the split hull and colonize between the kernel and coat, where the relative humidity is high enough to support spore germination (Mahoney and Rodriguez, 1996). Infected kernels can rapidly accumulate AFs under high relative humidity and temperature (Diener *et al*., 1987). More than 80% of pistachio nuts split before harvest (in some varieties up to 94%) (Tsantili *et al*., 2010), making the kernel more susceptible to *Aspergillus* invasion and aflatoxin contamination.

Even small numbers of spores can result in high levels of AFs (some times more than the permitted level) under favorable growth conditions. Aflatoxin contamination of pistachio nuts significantly reduces the quality and value of pistachios and directly affects farmers and consumers. Product recall and bans from international export are problems associated with high levels of aflatoxin contamination in pistachios (Doster and Michailides, 1994; Ellis *et al.*, 1991). According to the latest European Commission regulation, the maximum permitted level for nut products has been set at 2 ng/g for $AFB_1$ and 4 ng/g for total AFs (Pearson *et al.*, 1999; EC 1998; Sobolev, 2007).

AFs are chemically stable during processing and storage. It is critical to avoid the conditions leading to aflatoxin formation during pre-harvest, harvest, transport and storage, which is not typically possible or achieved in practice (Bullerman and Bianchini, 2007; Kabak, 2009). Although the aflatoxin level of agricultural products can be reduced by some chemical, biological and physical methods, it is almost impractical and very expensive to remove AFs after they are produced. Therefore, it is very important to prevent aflatoxin formation in the field. It has been reported that some microorganisms can be used as biocontrol agents against plant pathogens (Cook, 1993; Wilson and Wisniewski, 1989). Some yeasts have the capability to effectively compete with plant pathogens and can be used as biocontrol agents (Leibinger *et al*., 1997; Roberts, 1990; Yin, 2008). Saprophytic yeasts can be found on the surface of plant fruits and leaves. Under dry conditions, saprophytic yeasts colonize plant surfaces and produce extracellular polysaccharides to enhance their survivability. By producing extracellular polysaccharides, these yeasts restrict nutrient flow and inhibit the colonization of other microorganisms. Some researchers have used saprophytic yeasts to control *Aspergillus* in nut trees (Hua *et al.*, 1997, 1999; Hua, 2001; Palumbo *et al.*, 2006). The aim of this study was to isolate saprophytic yeasts from pistachio fruits and leaves and to investigate their biocontrol activity against *Aspergillus*.

## Materials and Methods

### *Fungal strain*

*A. flavus* PTCC 5006 (toxigenic strain) was used as a positive control to test the biocontrol activity of yeast isolates. *A. flavus* PTCC 5006 has been demonstrated to produce $AFB_1$. *A. flavus* PTCC 5006 was maintained on potato-dextrose agar slants (PDA). Water containing 0.05% Tween-80 was used to resuspend the fungal spores, and a hemocytometer was used to count the number of spores in the suspension.

### *Isolation of saprophytic yeasts*

"Ahmad aghaie" is one of the pistachio varieties that displays early splitting. Pistachio leaves and fruits were collected from Rafsanjan pistachio orchards (the first collection was during July and August 2004 while the second collection was during July and August 2005 (Afsah-Hejri *et al*., 2005). Samples were maintained at 4ºC in clean plastic bags, and the yeast isolations were performed within 24 h of sample collection. To isolate saprophytic yeasts, 40 g of pistachio leaves and 200 g of fresh fruits were separately added to 200 ml of sterile water containing 0.05% Tween-80 and placed on a rotary shaker (180 rpm) for 60 min. NYDA media (yeast extract 5 g/L, nutrient broth 8 g/L, dextrose 10 g/L, chloramphenicol 100 mg/L and agar 15 g/L) was used to spread 50 µL of suspension. NYDA plates were incubated for 48 h (28ºC). Yeast colonies were identified and streaked on Malt Extract Agar plates (MEA) (Barnett *et al*., 1990). Single and pure yeast colonies were then used for the remainder of the study.

### *Identification of saprophytic yeasts*

Saprophytic yeasts were identified based on their morphological properties and biochemical

tests. Isolated yeasts were stained and observed by microscopy. The following identification tests were performed.

*Urea hydrolysis test*

Urea broth (Difco, USA) was filtered using a 0.2 µm filter and inoculated with fresh yeast suspension before being incubated at 30ºC for 24 h. Yeast isolates were tested for urease enzyme activity.

*Glucose fermentation test*

Dextrose M and Yeast Extract (Merck, USA) were sterilized, inoculated with fresh yeast suspension and incubated at 30ºC for 48 h.

*Ascus production ability test*

MEA, YM agar and McClary agar (Weiqing, 1990) were used to test for the ascus production ability of the yeasts. Fresh yeast suspensions were inoculated on slants prepared from each media and incubated at 30ºC for 6 weeks. The Ziehl-Neelsen staining technique was used to observe the results.

*Potassium nitrate assimilation test*

Yeasts were tested for their ability to use nitrogen sources using Yeast Carbon Base agar (Difco, USA) (Pincus *et al*., 1988). A sterilized saturated potassium nitrate solution was used to prepare nitrate discs. Nitrate discs were placed on inoculated plates and incubated at 30ºC for 48 h.

*Carbon source utilization tests (auxanograms)*

To evaluate the ability of the yeasts to use different carbon sources (Barnett and Ingram, 1955), 1% solutions of 21 different carbon sources were used to prepare discs. Sterilized Yeast Nitrogen base agar (Difco, USA) was inoculated with fresh yeast suspensions. Discs were placed on inoculated plates, and the plates were incubated at 30ºC for 48 h.

*Exopolysacharide production test*

Yeast Extract base medium is a good medium to study the production of extracellular polysaccharides by yeasts (Christensen *et al.*, 1985). In this study, sterilized YM Broth (Difco, USA) was inoculated with fresh yeast suspensions and placed on a shaker (120 rpm) at 27ºC for 48 h. Iodine reagent was used to evaluate the extracellular polysaccharide production by the yeasts.

*Diazonium blue B test*

Diazonium blue B (DBB) is used to identify Basidiomycetous yeasts (Hagler and Ahearn, 1981). Ten-day-old yeast cultures on YM agar were used to react with the DBB.

*Growth at temperature higher than 30ºC (35ºC)*

Yeast isolates were inoculated on MEA slants and incubated at 35ºC for 1 week to test their ability to grow at a high temperature.

*Biocontrol test*

To study the biocontrol activity of the yeast isolates, a toxigenic strain of *A. flavus* (PTCC 5006) was used. Forty-eight-hour-old yeast isolates grown on YM broth were used to prepare spore suspensions of $10^7$ cells/ml in water containing 0.05% Tween-80. One-week-old toxigenic *A. flavus* grown on PDA was used to prepare spore suspensions. To generate 2 streaks of yeast inocula, 20 µl of a saprophytic yeast suspension ($10^7$ cells/ml) was applied 1 cm from the center of the PDA plates on each side of the centerline. After 4 h, 20 µl of the *A. flavus* (PTCC 5006) spore suspension ($10^7$ cells/ml) was applied on the centerline. The plates were then sealed and incubated for 10 days at 28ºC. Inoculated plates were observed visually for the antagonistic effects of the yeast isolates on *A. flavus* on days 4, 7 and 10. Growth inhibition was measured by reductions in biomass and the control of sporulation.

*Visual assay*

The plates were subjected to a visual assay. A score of "++++" was assigned to the positive control A. flavus (PTCC 5006) for a significant amount of mycelium production, and "-" represented a very small amount or an imperceptible level of growth.

*Sporulation control*

To evaluate the inhibitory effect of the yeast isolates on the sporulation of *A. flavus*, agar strips (1.2 cm width) were cut from the centerline of the PDA plates. The agar strips were transferred into bottles containing 20 ml of water and 0.05% Tween-80, and the bottles were shaken (150 rpm) for 1 h. A hemocytometer was used to count the fungal conidia.

*Biomass reduction assay*

To evaluate the inhibitory effect of the isolated yeasts on *A. flavu*s, the dry weight of the fungal mass was measured. The contents of the bottles from the previous step were boiled for 10 min. The hot suspension was then filtered using pre-weighed Whatman filter paper and washed with double distilled water. The filters were dried at 50ºC overnight before being weighed. Biomass production by *A. flavus* in the presence of each yeast isolate was measured.

*Analysis of aflatoxin production in media*

The PDA plate contents were transferred into

glass bottles containing methanol (85%) and shaken for 90 min on a rotary shaker (120 rpm). The suspension was then centrifuged at 3500 rpm for 20 min. The supernatant was used for aflatoxin analyses using reverse-phase High Performance Liquid Chromatography (HPLC) according to Iran National Standard No. 6872.

*Statistical analysis*

All measurements were performed in triplicate and are reported as the mean ± SD. Values with $p < 0.05$ were considered significant for the response variables. The results were subjected to a one-way analysis of variance (ANOVA).

## Results and Discussion

Twenty-four yeast isolates were obtained from pistachio fruits and leaves. The yeasts displayed different colony colors such as white, cream, beige, orange, light brown, pink and violet (Figure 1). Single yeast colonies were selected and observed under a microscope after staining (Figure 2), and 24 isolates were confirmed to be yeast. The confirmed yeast isolates were tested biochemically. During the biocontrol study, 5 of the yeast isolates displayed significant inhibitory activities against *A. flavus* growth (Figure 3), and AFB1 production was significantly reduced ($p < 0.05$).

As shown in Figure 3, the growth of *A. flavus* 5006 was restricted to and within the boundary of the 2 streaks of the saprophytic yeast zone. The *A. flavus* 5006 conidiophores grown in presence of the yeast isolates were less dense compared to the positive control. S.Y.76 was the most effective yeast and had the lowest visual score "-", followed by S.Y.91 and S.Y.81 with visual scores of "+" while S.Y.80 and S.Y.90 had visual scores of "++". The yeast isolates displayed significant inhibitory effects on the colony expansion of *A. flavus* 5006. As shown in Figure 3, the *A. flavus* 5006 colonies expanded towards the edges of the plate in the absence of the saprophytic yeasts. This visual assay is a good indicator of the antagonistic effect of saprophytic yeasts on *A. flavus* 5006 as its values are in agreement with the sporulation, dry weight and $AFB_1$ results (Figures 4, 5 and 6).

Figure 4 shows the significant effect ($p < 0.05$) of the saprophytic yeasts on reducing the spores produced by A. flavus 5006. *A. flavus* 5006 produced $325\times10^5$ spore/ml on PDA plates, whereas the number of spores was reduced to $50\times10^5$ in the presence of S.Y.76, indicating that conidia production was significantly reduced (between 62-85% for all 5 isolates) by the yeasts isolated from the pistachio fruits and leaves.

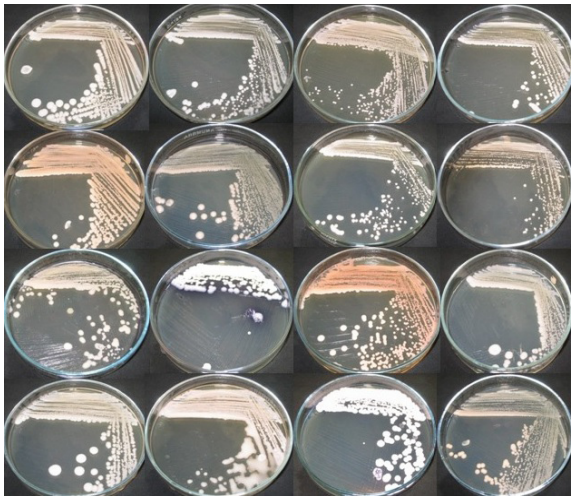

Figure 1. Colony colors of yeasts isolated from pistachio fruits and leaves

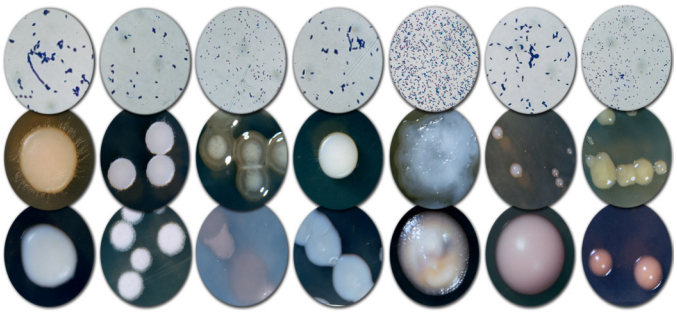

Figure 2. Yeasts isolated from pistachio fruits and leaves (down), single colonies after purification (middle), and stained yeast cells under the microscope (top)

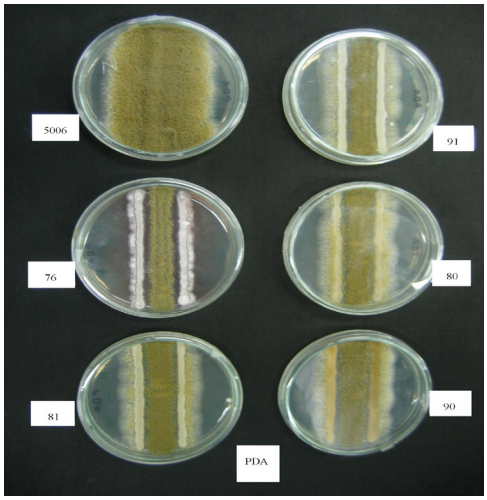


Figure 3. Growth inhibition of *A. flavus* 5006 by saprophytic yeasts isolated from pistachio fruits and leaves

Due to the inhibitory effects of the yeast isolates, the *A. flavus* 5006 dry weight was significantly ($p < 0.05$) reduced. S.Y.80 was able to lead to a 86% reduction in fungal biomass (Figure 5). The dry weight of *A. flavus* 5006 in the absence of the saprophytic yeast was 90 ± 0.07 mg per agar strip. S.Y.80 was the most effective yeast in terms of biomass reduction

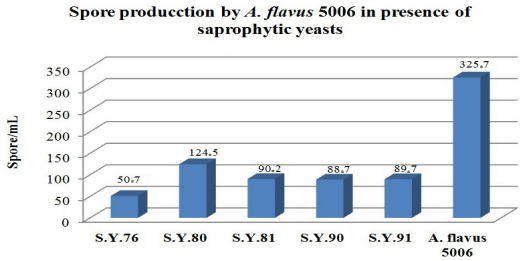


Figure 4. Effect of the yeast isolates on spore production by *A. flavus* 5006

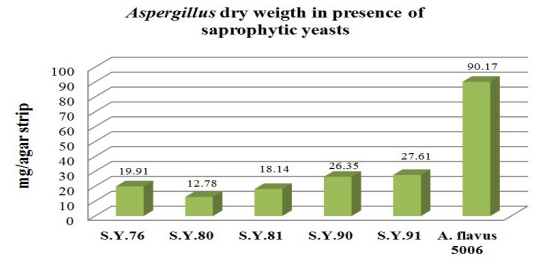


Figure 5. Effect of the yeast isolates on *A. flavus* 5006 dry weight

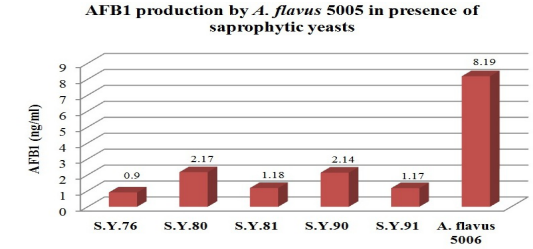


Figure 6. Effect of yeast isolates on AFB1 production by *A. flavus* 5006

followed by S.Y.81, S.Y.76, S.Y.90 and S.Y.91.

All 5 yeast isolates significantly ($p < 0.05$) inhibited AFB1 production by *A. flavus* 5006 (Figure 6). *A. flavus* 5006 produced 8.19 ng/ml of AFB1 in the absence of the saprophytic yeasts. Growing between 2 streaks of S.Y.76, AFB1 production by *A. flavus* 5006 was reduced to 0.9 ng/ml. S.Y.81 and S.Y.91 were more effective than S.Y.80 and S.Y.90 in terms of aflatoxin reduction. There was no significant difference between aflatoxin production in the presence of S.Y.81 or S.Y.91.

The results of the biochemical tests for the most effective yeast isolates are shown in Table 1. According to the results, all 5 selected yeast isolates were capable of hydrolyzing urea but were not able to ferment glucose. Except for S.Y.90, the remainder of the isolates used nitrate as a nitrogen source. None produced asci but all were positive in the DBB test, indicating that the strains belong to the basidiomycetes. To identify the isolated yeasts, Kurtzman identification keys were used (Kurtzman and Fell, 1998). S.Y.76 colonies were white in color on the front and blue to violet in color on the back of the colonies. Dimorphic colonies, abundant hyphae and fusiform conidia resulted in the identification

Table 1. Results of the biochemical tests for the identification of yeasts isolated from pistachio fruits and leaves

| | Ethanol | Methanol | Citrate | D-Gluconate | myo-Inositol | D-Mannitol | Glycerol | Starch | Raffinose | Lactose | Melibiose | Salicin | Cellobiose | Maltose | Sucrose | L-Rhamnose | L-Arabinose | D-Xylose | D-Ribose | D-Galactose | D-Glucose | Urea hydrolysis | Glucose fermentation | Nitrate | Ascus production | Exopolysaccharide | DBB |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SY.76 | + | - | + | + | + | + | + | - | + | - | - | + | +- | + | + | - | + | + | + | - | + | + | - | + | - | - | + |
| SY.80 | + | - | + | + | + | - | - | - | - | - | - | + | + | + | + | - | + | + | - | - | + | + | - | + | - | - | + |
| SY.81 | + | - | + | + | - | + | - | - | + | + | - | + | + | + | + | - | + | + | - | - | + | + | - | + | - | - | + |
| SY.90 | - | - | + | + | + | - | - | - | + | + | - | + | + | + | + | + | + | + | - | + | + | + | - | - | - | - | + |
| SY.91 | + | - | + | + | + | - | - | - | + | - | - | +- | + | + | + | +- | + | + | - | - | + | + | - | + | - | - | + |

of S.Y.76 as *Pseudozyma fusiformata*. According to the biochemical tests, S.Y.80 was identified as *Cryptococcus albidus* because it had bright white colonies with round cells without hyphae. S.Y.81 showed bright pink colonies, oval-shaped cells, no hyphal production and a specific fermentation pattern and was identified as *Rhodotorula fragaria*. S.Y.90 was identified as *Cryptococcus hungaricus* and produced pink colonies, whereas S.Y.91 was identified as *Rhodotorula hinula* due to its bright white cream colonies. The saprophytic yeasts isolated in this study have the potential for use as biocontrol agents against *Aspergillus* in pistachio orchards. Implementation of biocontrol methods will help to reduce the use of chemical compounds that affect both the environment and human health.

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